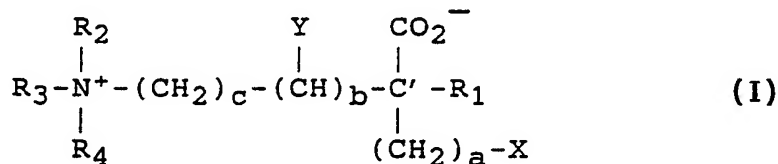




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**(54) Title:** DRIED BLOOD CELLS AND METHOD OF PREPARATION



**(57) Abstract**

A method for the drying of blood cells for rehydration, comprises incubating the cells to be dried in an aqueous medium containing one or more organic osmolytes before drying, optionally together with a polyol such as trehalose. The osmolytes are typically represented by formula (I), where a, b and c are each integers from 0 to 4; X is H, alkyl or NH<sub>2</sub>; Y is H, alkyl, or OH; R<sub>1</sub> and R<sub>2</sub> are each H or alkyl, or R<sub>1</sub> and R<sub>2</sub> together with N<sup>+</sup> and C' form a heterocyclic ring, e.g. a pyrrolidine ring; and R<sub>3</sub> and R<sub>4</sub> are each H or alkyl. Preferably the incubation medium contains substantially no potassium ion.

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## DRIED BLOOD CELLS AND METHOD OF PREPARATION

This invention relates to a process for the preservation of blood cells in the dried state such that they are stable to storage over long periods of time and which, when reconstituted, give biologically active cells.

Blood consists of an amber liquid termed plasma in which is suspended a number of formed elements - erythrocytes, leukocytes and platelets. In mammals the erythrocytes (red blood cells) have no nuclei and in humans they are biconcave discs of approximately 8 $\mu$ m diameter. They are produced continuously in the bone marrow and released in the circulation where they persist and function for a finite time (approximately 120 days in man) before being destroyed in the spleen. The major function of erythrocytes is to transport oxygen from the lungs to the tissues and transport carbon dioxide in the reverse direction, and this function is a product of the protein haemoglobin present in the cell. In combination with oxygen, haemoglobin forms oxyhaemoglobin, and in combination with carbon dioxide it forms carbamino haemoglobin.

The haemoglobin is contained within the cell by an elastic membrane composed of carbohydrates, lipids and proteins. The membrane acts as a selectively permeable barrier maintaining differences in the concentrations of sodium and potassium ions between the interior of the cell and the plasma, and also contains several active transport systems.

Platelets are small detached cell fragments or "minicells" derived from much larger cells called megakaryocytes. The megakaryocytes are produced in the bone marrow and remain there, the platelets starting their existence as vesicles within a megakaryocyte, before detaching themselves from its outer regions. The platelets are released into the circulation at the rate of approximately  $35 \times 10^9$  per day and having a lifespan of 9-10 days in the circulation. Platelets act to maintain the vascular integrity by sealing minor deficiencies in the endothelium, and promote vascular healing by the release of specific growth factor.

The plasma is a sticky, pale amber liquid which is a solution in water of proteins, salt and glucose, together with a variety of substances in transit. The proteins include albumin, which maintains the osmotic pressure of blood (although the other proteins also contribute to this), coagulation factors which

are responsible for maintaining the integrity of the vascular system, and the globulins which have a number of functions, including the carrying of immunity to infectious disease (the gamma-globulins).

The current practice for the collection and storage of blood requires that the blood is collected from healthy volunteers in FDA approved collection bags which contain a pre-measured amount of anti-coagulant. These standard collection bags are designed to receive 450ml of blood  $\pm$  10% and, provided that the sterility is maintained, the storage of the blood is limited only by the viability and stability of the individual components, which is determined by factors such as the temperature of storage and the anti-coagulant used.

Both erythrocytes and platelets are essential to normal well-being and there are a number of conditions resulting from injury, medical procedure or biochemical lesion which as a consequence lead to reductions in the levels of platelets or erythrocytes in the circulation. Such deficiencies must be corrected by transfusion. The transfusion of whole blood is justified only in rare cases following massive haemorrhage when both red blood cells and volume need to be replaced. It is more likely that a patient requires a specific fraction of blood to correct a particular disorder. Erythrocytes or plasma reduced blood is more suitable for correcting anaemia

occurring pre- and post-operatively. Platelets are recommended for patients with thrombocytopenia or an abnormality of platelet function or both, which is resulting in significant bleeding.

Whole blood stored under refrigeration using ACD (Acid-Citrate-Dextrose) is only available for transfusion for up to 21 days after collection. This expiry time corresponds to the time when 70% of these cells are still detectable in the circulation 24 hours after infusion. The use of CPD (Citrate-Phosphate-Dextrose) as an anticoagulant extends this "shelf life" to 28 days and with the addition of adenine to this anticoagulant, the life can be extended to 35-42 days.

Blood products stored below 0°C have a much longer shelf life but the conditions of storage are stringent. The length of permissible storage of blood products in the frozen state depends on factors such as the temperature of storage and the concentration of different additives within the preservation mixture.

Red blood cells suspended in a cryopreservative containing approximately 20% glycerol can be stored in liquid nitrogen for at least 3 years. Red cells

suspended in a cryopreservative containing a higher concentration of glycerol (approximately 40%) can be stored at  $-65^{\circ}\text{C}$  or lower for up to 15 years. As with refrigerated blood, freezers or liquid nitrogen containers have to be monitored continually to detect any increase in temperature, and must have the appropriate alarm system attached. Prior to transfusion, frozen red blood cells must be thawed, deglycerolised and then used within 48 hours.

Cold storage of blood and blood products, either at low temperatures in a liquid form, or at extremely low temperatures in the presence of a cryoprotectant has been the most favoured method of storage of blood since the First World War. The drawbacks with this method of storage, which until now have been insuperable, are obvious and are in the main related to the cost of the operation. Storage of blood for any length of time is at the present time geographically controlled by the presence of a power source to generate electricity to run the refrigerators, or by the availability of liquid nitrogen. Furthermore, centres of blood storage require expensive equipment (special refrigerators, pre-set alarms) for maintaining the blood in an optimum condition, and specially trained staff to manage these stocks to ensure their correct storage, rotation and use, and the maintenance of adequate supplies. Clearly,

in many of the poorer parts of the world, such complex and expensive storage facilities are simply not a viable option.

It would clearly be highly desirable to be able to store blood products in a form that was stable for long periods and did not need expensive and complex storage facilities. One option that has been explored extensively in the last few years is the possibility of desiccating blood products to give a dried form that would be stable for long periods at ambient temperature, and which could then be reconstituted by a simple rehydration procedure to give viable blood products that can then be used for diagnostic and therapeutic purposes. A number of recent patent applications, e.g. EP 0 367 468 and WO 90/04329, have described processes for the desiccation of mammalian cells to give dried products that on reconstitution are able to show much of their original antigenic activity. However, none of these desiccation processes have allowed the production of dried cell products that can be reconstituted to give viable, biologically active cells, and hence such products are wholly unsuitable for transfusion processes. Another application (EP 0 365 257) does actually claim a process for the freeze-drying (lyophilising) of mammalian cells in the presence of a



variety of monosaccharides, to give desiccated cells that can be reconstituted to give a small percentage of viable cells. This patent (EP 0 356 257) shows no Examples of the preservation of surface antigenicity, neither does it describe any long term storage experiments. Inability to fulfill both criteria would make such blood products unsuitable for therapeutic use.

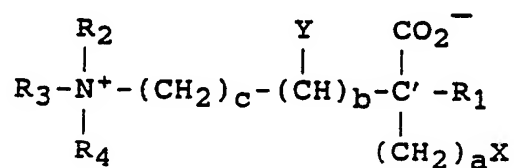
Thus it can clearly be seen that there is still an urgent need for a method of production of dried blood products in a form stable over long periods, that do not require expensive storage facilities, and which can be reconstituted by a simple rehydration procedure to give suspensions for transfusion containing a high percentage of viable cells i.e. a percentage of cells within the limits normally tolerated by the body. It is the object of this invention to provide such a method.

We have found that it is possible to dry blood cells in such a way as to produce dried cell products that can be stored for long periods at ambient temperature, and which on rehydration give aqueous suspensions in which a high percentage of the reconstituted cells are intact and still in possession of their original biological activity i.e. they are able to perform their normal biological functions. Hence, these reconstituted blood products may be utilised in a wide range of diagnostic and therapeutic applications.

It is known that in order to cope with cycling water stress a phylogenetically diverse group of organisms (bacteria, unicellular algae, vascular plants, invertebrates and some vertebrates) utilise a group of low molecular weight organic molecules (polyhydric alcohols, polyols, amino acids and amino acid derivatives) as organic osmolytes. The external environments which confront the cells of these organisms may change in terms of their water availability, and this in turn can lead to damage to macromolecules within the cells. In order to protect these macromolecules the cells accumulate intracellularly one or more organic osmolytes which balance the osmotic strength of the cytoplasm with that of the environment (see, for example, Le Rudulier et al, Science, 224, 1064-1068, 1984). It is wholly surprising and quite without precedent, however, that incubation of cells in solutions of such osmolytes should enable one to dry biologically active cells, which can then be stored for long periods at ambient temperature before rehydrating to give a high percentage of reconstituted cells that retain their original biological activity.

According to this invention we provide a method of drying blood cells for rehydration, characterised in that the cells to be dried are incubated in an aqueous system containing one or more organic osmolytes before drying.

In general, the blood cells are incubated in an aqueous solution containing one or more organic osmolytes at ambient temperature or above, the organic osmolyte(s) being present in any effective amount. Generally a concentration of the organic osmolyte(s) in the final incubation mixture of from 2 to 25% (w/v) is desirable, typically 10 to 20% e.g. 15%. The organic osmolytes are chosen from a group of low molecular weight organic molecules (e.g. molecular weights from about 80 to 180), that includes amino acids and amino acid derivatives. Preferred osmolytes are those represented by the formula



where a, b and c are each integers from 0 to 4; X is H, alkyl, or NH<sub>2</sub>; Y is H, alkyl, or OH; R<sub>1</sub> and R<sub>2</sub> are each H or alkyl, or R<sub>1</sub> and R<sub>2</sub> together with N<sup>+</sup> and C' form a heterocyclic ring, e.g. a pyrrolidine ring; and R<sub>3</sub> and R<sub>4</sub> are each H or alkyl. All alkyl groups in the above formula are lower alkyls i.e. C<sub>1</sub> - C<sub>4</sub>. Among these preferred osmolytes, carnitine, proline, and betaine are particularly preferred. After drying, the blood product can be stored dry at ambient temperature, with no significant deterioration over time.

Apart from the haemoglobin inside the erythrocyte, it is important for full effective functionality that the proteins and glycoproteins of the lipid membrane should also be preserved as some of these ensure the erythrocytes remain within the correct circulatory pathways for their normal lifespan. Thus, for clinical use, especially where a reasonably long half life is expected for the blood cells, it is necessary to preserve the components of the lipid membrane.

To this end, in addition to the organic osmolyte(s), the aqueous medium should desirably further contain a polyol preservative for the cell-wall antigens, namely a preservative disaccharide or trisaccharide or a non-reducing glycoside of a polyhydroxy compound selected from sugar alcohols and other straight chain polyalcohols.

The particularly preferred preservative is the non-reducing sugar trehalose, but other compounds of use include maltose and raffinose, and maltitol, lactitol and palatinit, the sugar alcohol from isomaltulose. Saccharides are preferably non-reducing. The concentration of the polyol in the final incubation mixture may be from 1 to 20% (w/v), particularly 5 to 20% e.g. 10%. We have found that for certain cell-types, the additional presence of trehalose in the

incubation mixture results in an increase in the percentage of cells able to perform their normal biological function after rehydration, compared to that achieved using organic osmolyte(s) alone. Conversely, however, we have shown that vacuum-drying of blood cells in the presence of trehalose, but in the absence of an organic osmolyte, produces a dried product that on rehydration gives only a very small percentage of cells.

The addition of polyols such as sugars and sugar alcohols appears to have additional advantages. Thus, in the presence of such a polyol, the cell structure appears to be strengthened, resulting in a higher proportion of intact cells surviving. Also the colour of the erythrocytes is preserved, seemingly with increased stability of the haemoglobin inside the cells. Further, the incubation itself is desirably effected at low temperature, typically a refrigerator temperature, e.g. about 4°C. We find that this is preferable to incubation at higher temperatures, e.g. about 37°C, since the osmolyte uptake and retention in the cell is maximised.

The suspension of incubated cells is preferably dried in vacuo. Freeze drying is not essential and, indeed, it is preferred to dry in vacuo at ambient temperatures, e.g. 4 to 30°C. The drying can conveniently be effected

by spreading the suspension into a thin film, for example in a rotary evaporator.

The method of reconstitution of the dried blood cells varies depending on the nature of the cell. In the case of erythrocytes, which have very delicate membranes, rehydration may be achieved using a medium hypertonic with respect to the tonicity of the cells in their normal, biologically active state (e.g. a solution of phosphate buffered saline containing glucopyranosyl sorbitol at a concentration of 40%), to give an aqueous suspension of the reconstituted cells. The rehydration medium may advantageously include potassium ions. A particularly preferred rehydration medium comprises a phosphate buffered saline containing  $K^+$  ions and an osmolyte, especially carnitine, at about 5 to 25 g/l, and polyethylene glycol (preferred MW approx 10,000) at about 50 to 70 g/l. The reconstituted cells may then be brought to tonicity over a period of time by gradual dilution of the mixture. Platelets, however, are much smaller cells with tougher membranes, and rehydration may be achieved by the addition of water to the same volume as before drying.

Erythrocytes incubated in the presence of proline and trehalose before drying may be reconstituted to give up to 98% apparently intact cells by microscopic examination.

Platelets incubated in the presence of osmolyte(s), optionally in the presence of a polyol such as trehalose, before drying, can be reconstituted by the addition of an equivalent volume of water to that which was present before dehydration to give an aqueous suspension of cells in which the number of biologically viable cells is sufficient for therapeutic use. Rehydration with an equivalent volume of water additionally containing 2% carnitine, however, gives results generally 25% better than with water alone. Normal platelets agglutinate when triggered by ADP and the above reconstituted cells, on incubation in the presence of ADP, also show good agglutination times.

A major problem in many therapeutic situations is that of sterility, e.g. treatment of wounded on the battlefield. It can be readily envisaged that it would be possible to devise a two-part sterile plastic container, in which the two halves were separated by a breakable seal. One half of the container would hold the dried blood product, while the other would hold the rehydrating medium. Simply breaking the seal would then enable the blood product to be reconstituted immediately whenever and wherever it was required.

The following Examples illustrate the invention further. All percentages are expressed as weight per

volume, where the volume is the final volume of the mixture at the particular stage of the process being described.

Example 1    Effect of Concentration of Organic Osmolytes  
on the Preservation of Erythrocytes

Whole blood was collected by venepuncture into the anticoagulant EDTA (used at a final concentration of 5mM), and then incubated for 2 hours at 37°C in one of the following osmolytes: proline, carnitine and betaine, over a range of concentrations. The samples were then frozen into a thin shell on the walls of the vessel and then placed under vacuum for drying. The vacuum was typically less than 25  $\mu$ m Hg (33.3 Pa), and the samples were held under this vacuum for approximately 16 hours.

The freeze-dried samples were rehydrated by resuspending some of the dry material in an hypertonic solution consisting of phosphate buffered saline (PBS) containing glucopyranosyl sorbitol at a concentration of 40%. The samples were then assessed microscopically to determine the number of intact erythrocytes.



The results are summarised in Table 1:

Table 1

<u>Organic</u> <u>Osmolyte</u>	<u>Final</u> <u>Concentration</u>	<u>% Intact</u> <u>Cells</u>
Proline	5	0
	10	62
	15	81
	20	< 1
Carnitine	5	0
	10	< 1
	15	75
	20	62
Betaine	0	0
	5	0
	10	35
	15	< 5
	20	0

From the results above, freeze drying of erythrocytes in the presence of 15% proline or 15% carnitine can be seen to be particularly successful.

Example 2    Effect of a Different Rehydration Mixture

The dry powder from the two most successfully freeze-dried erythrocyte samples in Example 1 (i.e. dehydration in the presence of 15% proline and 15% carnitine) was taken and rehydrated with solution A (10% foetal bovine serum, 2% carnitine, 7.5% polyethylene glycol [mw 6000], suspended in PBS). After the dehydrated samples were resuspended fully, an equivalent amount of PBS was added, and the integrity of the erythrocytes were assessed microscopically.

The results are as summarised in Table 2:

Table 2

<u>Sample</u>	<u>% Intact Cells</u>
15% proline	79
15% carnitine	87

Example 3    Effect of the Additional Presence of  
Trehalose in the Incubation Mixture Before  
Freeze-Drying

Blood was collected by venepuncture into the anticoagulant EDTA (used at a final concentration of 5mM), and then incubated for 2 hours at 37°C in either

10% or 15% proline plus trehalose over a range of concentrations. The samples were then frozen into a thin shell on the walls of the vessel and then placed under vacuum for drying. Freeze-drying was then carried out as in Example 1.

The freeze-dried samples were then rehydrated by resuspending some of the dry material in solution consisting of PBS containing glucopyranosyl sorbitol at a concentration of 40%. The samples were then assessed microscopically to determine the number of intact erythrocytes.

The results are summarised in Table 3:

Table 3

Final Concentration of Proline and Trehalose	% Intact Cells
10% proline, 5% trehalose	68
10% proline, 10% trehalose	80
10% proline, 15% trehalose	87
15% proline, 5% trehalose	74
15% proline, 10% trehalose	92

From these results it can be seen that up to 92% intact cells can be attained on rehydration.

Example 4    Survival of Biologically Functional  
Haemoglobin Within Dried Erythrocytes

Blood was withdrawn by venepuncture into the anticoagulant EDTA (at a final concentration of 5mM) and then aliquots of the blood were incubated with the following additives:

1.     15% proline
2.     15% proline, 10% trehalose
3.     15% carnitine
4.     15% carnitine, 10% trehalose

The solutions were incubated at 37°C for 2 hours and then frozen onto the walls of a pyrex glass flask in a thin shell by first immersing the flask while rotating it, in a mixture of solid carbon dioxide and methylated spirits. After the contents had solidified onto the walls, the flask was immersed in liquid nitrogen, and then attached to a freeze drying machine so that the contents of the flask was subjected to a vacuum of less than 25  $\mu$ m Hg (33.3 Pa). The blood was dried under this vacuum for at least 16 hours. When dry the blood had a crystalline appearance.

The dry blood was rehydrated with 1 volume of solution A (see Example 2), and when all the dry blood was resuspended, an equivalent volume PBS was added. The number of intact cells was assessed by fluorescein dye exclusion, the results being summarised in Table 4:

Table 4

<u>Additive Used in Drying</u>	<u>% Intact Cells</u>
15% proline	78
15% proline, 10% trehalose	82
15% carnitine	70
15% carnitine, 10% trehalose	74

The samples were then washed 3 times, by centrifugation and resuspension, with a solution made from mixing equivalent volumes of solution A and PBS. The cell pellet was then resuspended in 9 volumes of water to lyse the erythrocytes, and then 1 volume of 10X concentrated PBS was added.

The samples were then taken and treated as follows:

(1) gaseous oxygen was bubbled through the samples for 5 to 10 minutes, and then the absorption spectrum between 450nm and 700nm was recorded;

(2) gaseous carbon dioxide was then bubbled through the sample for 5 to 10 minutes, and then the absorption spectrum between 450nm and 700nm was recorded;

(3) gaseous oxygen was then bubbled through the samples for 5 to 10 minutes, and then the absorption spectrum between 450nm and 700nm was recorded.

The spectra from this experiment are as shown in Figures 1(a) to 1(f) of the accompanying drawings, the samples whose spectra are shown being as follows:

(a) Normal undried blood, which was washed by centrifugation and resuspension in PBS. The cell pellet was resuspended in 9 volumes of water to lyse the erythrocytes, and 1 volume of 10 times concentrated PBS was added.

(b) Erythrocytes dried in 15% proline and subsequently rehydrated.

(c) Erythrocytes dried in 15% proline and 10% trehalose and subsequently rehydrated.

(d) Erythrocytes dried in 15% carnitine and subsequently rehydrated.

(e) Erythrocytes dried in 15% carnitine and 10% trehalose and subsequently rehydrated.

(f) Erythrocytes dried without additions and subsequently resuspended in phosphate buffered saline.

On each sample the absorption spectra are labelled 1, 2 and 3:

1. Spectrum following initial pulse with oxygen.
2. Spectrum following pulse with carbon dioxide.
3. Spectrum following final pulse with oxygen.

It is clear from these results that the ability of the intracellular haemoglobin to function in reversibly combining with oxygen or carbon dioxide, (as shown by the changes in the absorption spectrum), is substantially wholly preserved provided that the blood was incubated in the presence of an organic osmolyte before freeze drying. Of the incubation mixtures tested above, the best preservation of both cell number and haemoglobin function would appear to be with 15% proline and 10% trehalose.

#### Example 5    Freeze-Drying of Platelets

Blood was collected into the anti-coagulant, acid-citrate-dextrose, and the mixture then centrifuged at approximately 1100 x g for three minutes to harvest Platelet Rich Plasma.

Aliquots of the Platelet Rich Plasma were then incubated in the presence of varying concentrations of proline, carnitine and trehalose on their own, and proline and carnitine in combination with trehalose, as well as a

test sample in 7.5% NaCl. All samples were incubated for 37°C for 2 hours on a slowly moving rotary mixer, before freezing and drying.

The dehydrated samples were rehydrated with either 1 volume of water, or 1 volume of water containing 2% carnitine. It was found that for those platelets incubated in the presence of carnitine or proline, or either of these osmolytes combined with trehalose, the percentage of whole platelets recovered was between 60 and 95%. The best results were achieved with 15 % proline + 5% trehalose, and 15% carnitine + 10% trehalose. Platelets incubated in the presence of trehalose alone showed almost exclusively 'ghost' platelets when reconstituted. Results obtained when rehydrating with water in the presence of 2% carnitine were generally about 25% better than those achieved with water alone.

#### Example 6    Aggregation Properties of Reconstituted Platelets

Platelet-rich plasma (PRP) was obtained by collecting blood into citrate buffer and centrifuging, e.g. at approximately 1100 x g for 3 minutes, to give a supernatant containing platelets and a precipitate containing other cells. A sample was freeze-dried in the presence of 15% w/v proline 10% w/v trehalose 10%



carnitine and 5% glucose and subsequently rehydrated to its original volume with water and subjected to aggregation by addition of ADP (a 1 mg/10 ml stock solution) at a dilution of 1:10. The results were compared with those for fresh PRP by measuring optical density changes. A significant degree of aggregation was noted.

### Example 7

#### 1. Collection and Dehydration

Fresh blood was withdrawn by venepuncture from healthy volunteers into the anti-coagulant EDTA (final concentration 5mM). The blood was centrifuged and then washed 3 to 4 times in a potassium free buffered salt solution which contains the following ingredients:

Table 5

Compound	Amount (g/l)
NaCl	8.12
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	3.6
NaH <sub>2</sub> PO <sub>4</sub> · 2H <sub>2</sub> O	0.8
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.15
Proline	150
Trehalose	100

The cells were then resuspended finally at 50% and incubated for 60 minutes at 4°C, and aliquots were then dried at ambient temperature (evaporation cooled the samples) under a vacuum of less than  $5 \times 10^{-2}$  mbar (50 Pa) for approximately 16 hours. At this stage the material is very crisp and can be powdered easily.

## 2. Rehydration

Rehydration of the blood was accomplished using 2 solutions, the contents of which are listed in the following Tables.

### Solution 1

### Table 6

Compound	Amount (g/l)
NaCl	8.0
KCl	0.4
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.6
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.8
Glucose	2
Casein Hydrolysate <sup>1</sup>	10
Carnitine	20
PEG <sup>2</sup>	60

Solution 2Table 7

Compound	Amount (g/l)
NaCl	8.0
KCl	0.4
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.6
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.8
Glucose	2
Casein Hydrolysate <sup>1</sup>	5
Carnitine	10
PEG <sup>2</sup>	30

1. NZ-Amine Types AS Enzymic Digest of Casein, High Solubility (Morham Ltd, East Sussex).

2. Polyethylene Glycol (MW 10,000)

The red cells were dried in aliquots of 250 $\mu$ l and the rehydration was initiated by the addition of 250 $\mu$ l of solution 1 to the dry aliquots. The cells were allowed to rehydrate slowly, and when in solution, 500 $\mu$ l of solution 2 was added. At this stage the cells were viewed under the microscope and the number of apparently intact cells was counted. A further 1 ml of solution 2 was then added and the cells were then centrifuged at 5,220 x g for 5 minutes. The supernatant was removed and

the cells were resuspended in solution 2. For typing the red cell antigenic status, an equal volume of the red cells in solution 2 was added to an equal volume of an antibody mixed 1 : 1 with solution 1. The normal times of protocols for typing with each antibody was then followed, and the results were scored according to "Reading and Grading of Hemagglutination Reactions" (Ortho Blood Bank Educational Series). The results are shown in Table 8.

Table 8

Erythrocytes phenotyped as  $AR^1rNNK_1^-Fy^{a-b+}$  were dried and their antigenic status was tested after storage at room temperature for:

Antibody versus blood group	Fresh blood	1 day	2 weeks	1 month	2 months
A	4+	4+	4+	4+	4+
B	-	-	-	-	-
Rh D	3+	3+	3+	3+	3+
Rh c	2+	2+	2+	2+	2+
Kell	-	-	-	-	-
M	-	-	-	-	-
N	3+	4+	4+	4+	4+
Duffy ( $Fy^b$ )	2+	+	2+	2+	2+
% of apparently intact cells	>98%	>98%	>98%	>98%	>98%

Example 8    Effect of different sugars and sugar alcohols  
on storage.

A series of dried samples were prepared in a method similar to that in Example 7 but in which the sugar component of the washing and incubation media used prior to dehydration was varied. The concentration of each sugar used was 10% (w/v). The blood was dried in aliquots of 250µl under a vacuum as in Example 7 and then stored at room temperature in a plastic bag with a crimped strip seal, in the presence of a pouch of desiccant. After 3 months the cells were rehydrated according to the protocol described in Example 7 and then tested to determine their antigenic profile.

The results are shown in Table 9 below. It would appear from these results that the integrity of the cell in the dry state and on rehydration can be maintained by the osmoprotectant, proline, alone. However there are three important points to note, the first is that where proline was used as the sole protective agent there appeared to be some deterioration in the sample. On rehydration the samples which contained proline alone were brown/black in colour which would indicate that the cell and/or its contents may have undergone degeneration possible due to some form of reduction reaction.

However in the presence of trehalose, maltose, maltitol,

TABLE 9

Results of rehydration of red cells dried in different sugars and stored at room temperature for 3 months

Proline plus the following sugars	Colour on rehydration	% apparently intact cells	Agglutination patterns with antibodies against				
			A	B	RhD	Rhc	Kell
NO SUGAR	Brown/black	67%	4+	-	4+	+	-
SORBITOL	Brown	15%	4+	-	4+	+	-
MALTOSE	Red	80%	4+	-	4+	2+	-
TREHALOSE	Red	74%	4+	-	4+	2+	-
MALTITOL	Red	82%	4+	-	4+	2+	-
LACTITOL	Red	86%	4+	-	4+	2+	-
PALATINIT	Red	94%	4+	-	4+	2+	-
RAFFINOSE	Red	90%	4+	-	4+	+	-

lactitol palatinit or raffinose, the samples appeared to be the normal bright red colour.

The second point is that in the absence of a sugar there appears to be a loss of antigenicity, which is apparent as a reduction in the amount of agglutination seen with anti-Rhesus c antibody. Thus over this storage period many sugars appear to work effectively at preserving the antigenicity of the cells and their integrity.

The third point is that the sugar alcohols appear to help stabilize the cell structure itself, as evidenced by the percentage of intact cells obtained.

#### Example 9 Quantitation of mean cell haemoglobin levels

##### a) Collection and drying of blood.

Blood was taken from a volunteer by venepuncture into the anticoagulant EDTA (final concentration 5mM). A sample of this blood was taken, centrifuged, and washed in potassium free balanced salt solution (as in Table 5) then incubated at 4°C for 60 minutes in potassium free balanced salt solution supplemented with 10% trehalose and 15% proline. The blood was then dried in aliquots of 250 $\mu$ l under a vacuum of less than  $8 \times 10^{-2}$  mbar (80 Pa) for more than 16 hours.

## b) Rehydration of blood

The cells were rehydrated using 2 solutions. Solution 1 as in the following Table, and solution 2 as in Example 8.

Compound	Amount per litre
KCl	10.4g
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8mg
Vitamin C	7mg
MgCl <sub>2</sub> ·6H <sub>2</sub> O	418mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	4mg
Calcium lactate	218mg
Tris buffer pH7.2	10mM
Casein Hydrolysate <sup>1</sup>	10g
Carnitine	20g
PEG <sup>2</sup>	75g

1 and 2 : As in Example 8

The red cells were dried in aliquots of 250µl and the rehydration was initiated by the addition of 250µl of solution 1 to the dry aliquots. The cells were allowed to rehydrate slowly, and when completely in suspension, a further 500µl of solution 2 was added. To this was added an additional 1ml of solution 2 and the cells were then centrifuged at 5,220 x g for 5 minutes.

The supernatant was removed and the cells were resuspended in solution 2 and counted. c) Release of haemoglobin and its measurement.



The method used for the release of haemoglobin and its measurement was the same irrespective of whether the sample was fresh blood or rehydrated blood. For fresh blood the blood was resuspended in phosphate buffered saline, counted and centrifuged at  $1,310 \times g$  for 10 minutes. The cells were then lysed to release the haemoglobin. For the rehydrated blood in solution 2, the cells were centrifuged at  $5,220 \times g$  for 5 minutes and then lysed to release their haemoglobin.

The cells were lysed by resuspension in 1ml of deionised, distilled water. The samples were then agitated after which 250 $\mu$ l of carbon tetrachloride was added and the solution was vortexed and centrifuged at  $16,000 \times g$  for 10 minutes. The free haemoglobin in the supernatant was determined spectroscopically.

#### Results:

Sample	MCHb <sup>1</sup> (pg/cell) $\pm$ SD (n)	% of fresh control
Fresh erythrocytes	27.75 $\pm$ 5.46 (n=4)	100%
Erythrocytes incubated in trehalose and proline, dried and rehydrated	16.87 $\pm$ 3.26 (n=4)	61%

1. Mean cell haemoglobin level

Results from a similar experiment, this time replacing the trehalose with other sugar alcohols:

Sample	MCHb(pg/cell) $\pm$ SD	% of fresh control
Fresh erythrocytes	30.43 $\pm$ 3.9 (n=4)	100%
Erythrocytes incubated in glucopyranosyl mannitol and proline, dried and rehydrated	18.68 $\pm$ 9.94 (n=6)	61%
Erythrocytes incubated in glucopyranosyl sorbitol and proline, dried and rehydrated.	24.40 $\pm$ 3.34 (n=4)	80%

Example 10 Experiment to show that haemoglobin from red cells stored on the bench for 2 months can reversible bind oxygen and carbon dioxide.

The red cells were collected and dried in a manner similar to that described in Example 7. The dried red cells were stored at room temperature in a ziplock bag containing pouches of desiccant. The samples were rehydrated approximately 2 months later in a manner similar to that described in Example 7. The red cells

were then lysed with distilled water to release the intracellular haemoglobin. One volume of ten times concentrated phosphate buffered saline was added to 9 volumes of haemoglobin in distilled water, and the samples were then treated as follows:

(i) the spectrum of the sample was read over the range 450nm to 700nm.

(ii) oxygen was then bubbled through the sample for approximately 5 minutes, and the spectrum was read over the range 450nm to 700nm (Figure 2, line 1).

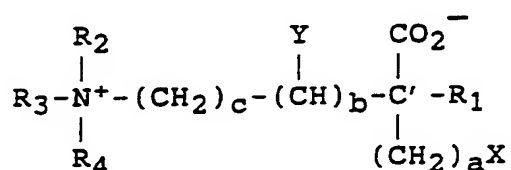
(iii) carbon dioxide was then bubbled through the sample for about 5 minutes, and the spectrum again read over the same range (Figure 2, line 2).

(iv) oxygen was then bubbled through the same for approximately 5 minutes and the spectrum again read over the same range (Figure 2, line 3).

The changes in the spectrum of the haemoglobin from the sample are substantially similar to that seen with haemoglobin from fresh red cells shown in Example 4.

CLAIMS

1. A method of drying blood cells for rehydration, characterised in that the cells to be dried are incubated in an aqueous system containing one or more organic osmolytes before drying.
2. A method according to Claim 1, in which the organic osmolytes have a molecular weight from 80 to 180 and are selected from amino acids and amino acid derivatives.
3. A method according to Claim 1, in which the organic osmolytes are represented by the formula



where a, b and c are each integers from 0 to 4; X is H, alkyl or NH<sub>2</sub>; Y is H, alkyl, or OH; R<sub>1</sub> and R<sub>2</sub> are each H or alkyl, or R<sub>1</sub> and R<sub>2</sub> together with N<sup>+</sup> and C' form a heterocyclic ring, e.g. a pyrrolidine ring; and R<sub>3</sub> and R<sub>4</sub> are each H or alkyl.

4. A method according to Claim 3, in which the organic osmolytes are selected from proline; carnitine; and betaine.

5. A method according to any of Claims 1 to 4, in which the amount of the organic osmolyte(s) in the incubation mixture is from 2 to 25% (w/v).
6. A method according to Claim 5, in which the amount of the organic osmolyte(s) in the incubation mixture is from 10 to 20%(w/v).
7. A method according to Claim 5 or Claim 6, in which the organic osmolyte is proline or carnitine, and is present in the incubation mixture at about 15% (w/v).
8. A method according to any of Claims 1 to 7, in which the incubation mixture additionally contains a preservative polyol.
9. A method according to Claim 8 in which the polyol is a preservative di- or tri- saccharide or a non-reducing glycoside of a poly hydroxy compound selected from sugar alcohols and other straight chain polyols.
10. A method according to Claim 9, in which the amount of polyol in the incubation mixture is from 1 to 20% (w/v).

11. A method according to any of Claims 1 to 10, in which the polyol is trehalose.
12. A method according to any of Claims 1 to 11, in which the incubation is effected at low temperature.
13. A method according to any of Claims 1 to 12, in which the incubation mixture additionally contains an anti-coagulant.
14. A method according to Claim 11, in which the amount of trehalose in the incubation mixture is 10% (w/v) and the amount of proline or carnitine is 15% (w/v).
15. A method according to any of Claims 1 to 14, in which dried erythrocytes are rehydrated with a medium hypertonic to the tonicity of the erythrocytes in their normal, biologically active state.
16. A method according to Claim 15, in which the rehydration is carried out with a phosphate buffered saline solution containing an osmolyte and polyethylene glycol (MW 10,000).
17. A method according to any of Claims 1 to 14, in

which dried platelets are rehydrated by the addition of an equivalent amount of water to that which was present before dehydration.

18. Dried blood cells for rehydration, characterised in that the blood cells have been dried by a method according to any of Claims 1 to 15.
19. Blood cells, characterised in that the cells have been dried and subsequently rehydrated by a method according to any of Claims 1 to 18.
20. Blood cells according to Claim 19, in which the blood cells are erythrocytes.
21. Erythrocytes according to Claim 20, in which greater than 75% of the cells are intact.
22. Erythrocytes according to Claim 21, in which up to 98% of the rehydrated cells are intact.
23. Blood cells according to Claim 19, in which the blood cells are platelets.
24. A rehydrated blood product obtained by rehydrating dried blood products according to Claim 18.

1/3

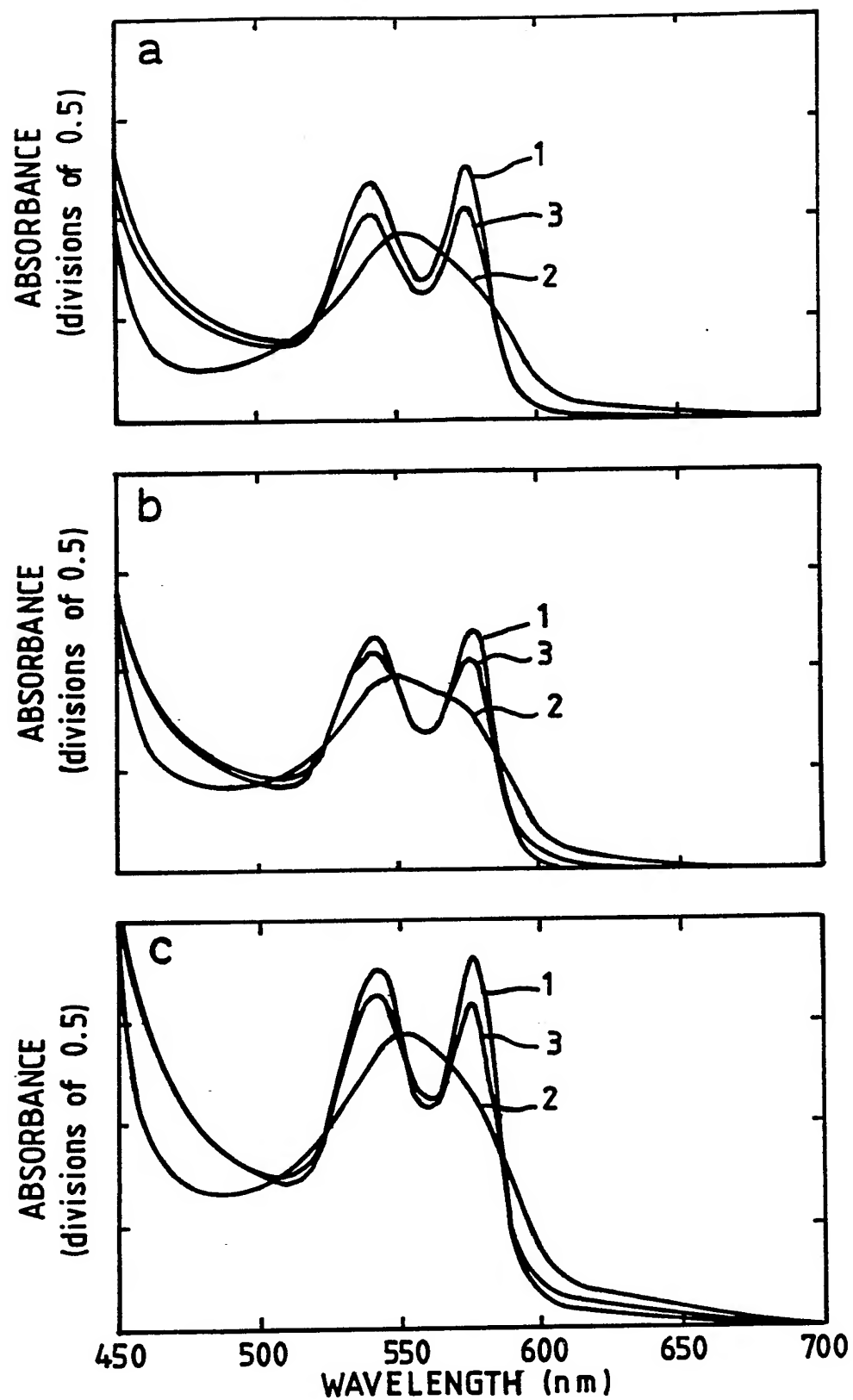


FIG.1



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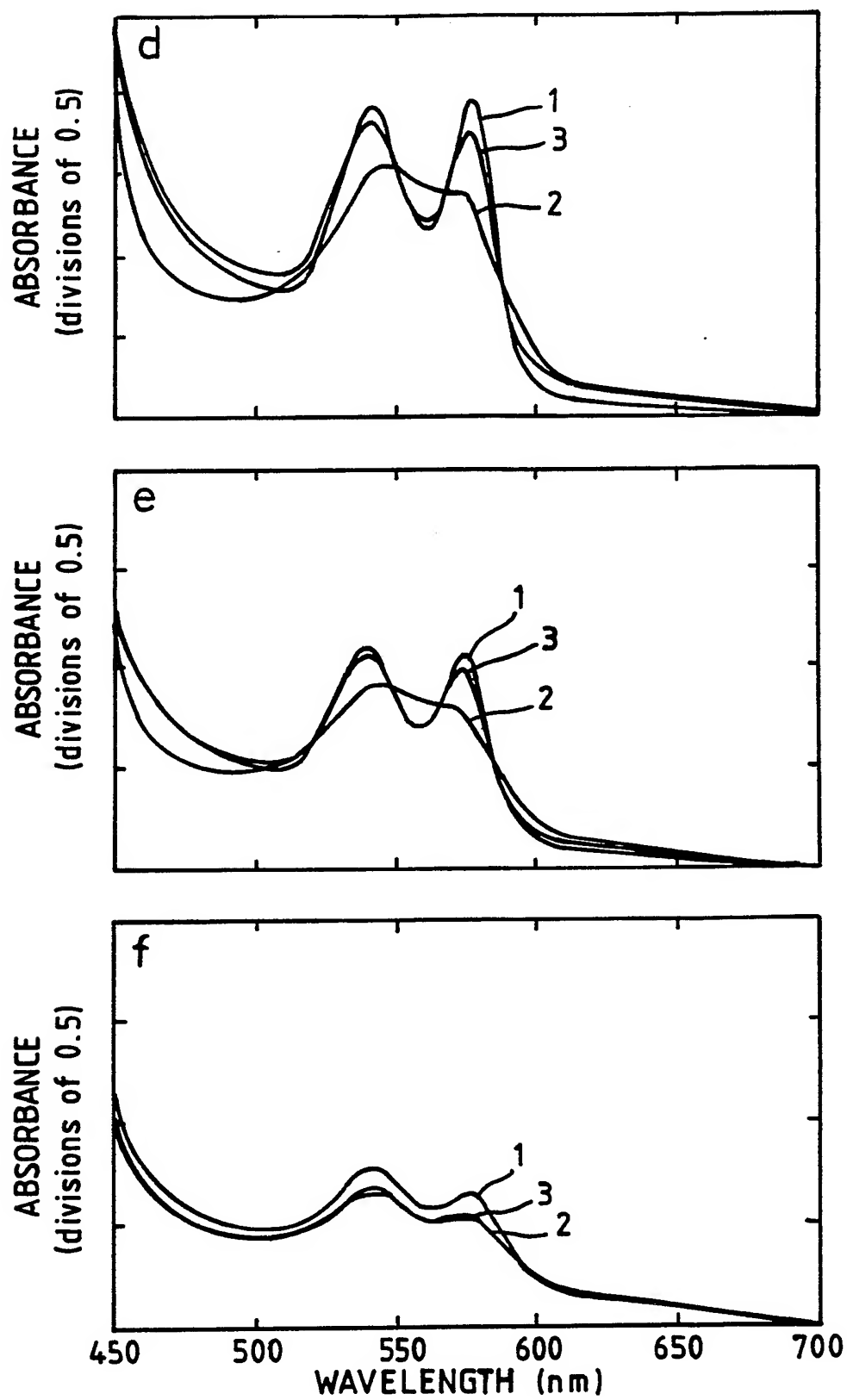


FIG.1(contd.)

3/3

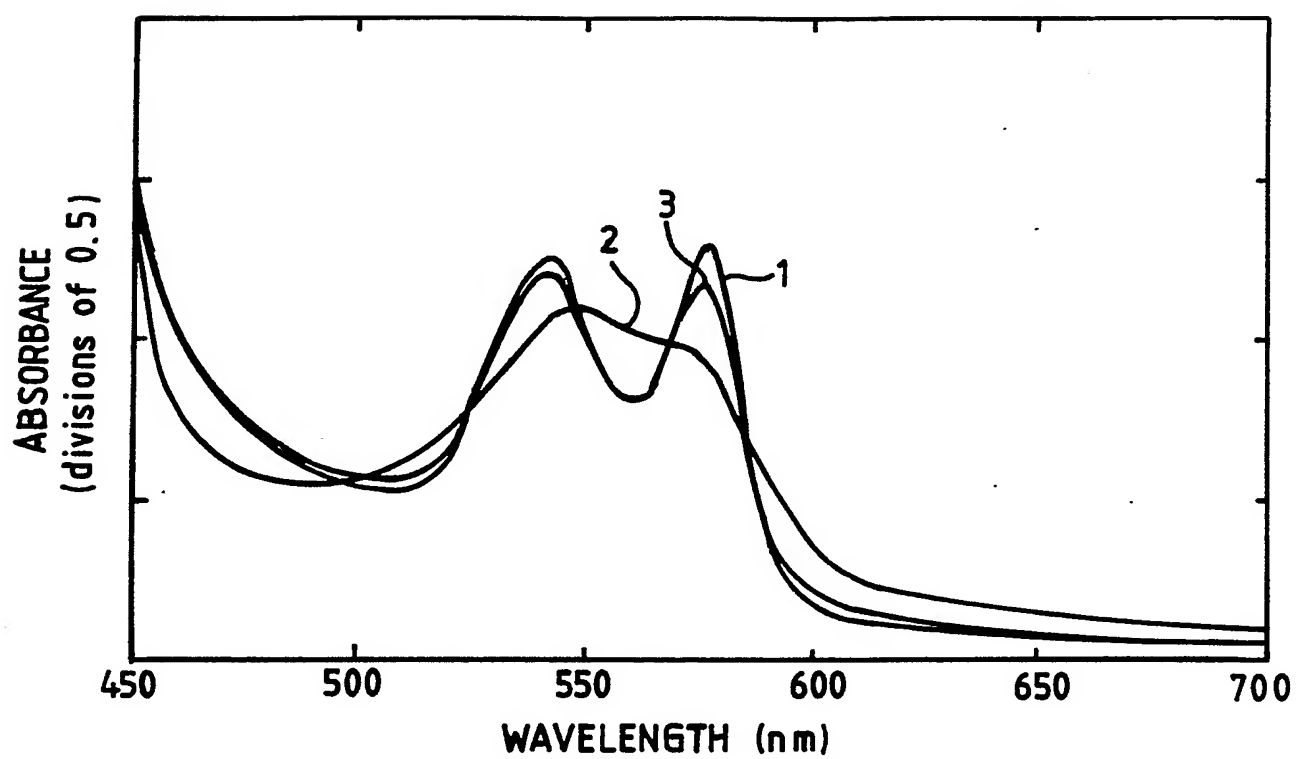


FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 92/01183

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A01N1/02

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl. 5

A01N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X,P	Week 9212, Derwent Publications Ltd., London, GB; AN 92-096488 US PATENT APPLICATION 7659765(RUDOLPH A. ET AL.) PUBLISHED 4 FEBRUARY 1992 see abstract	1-24
A	EP,A,0 246 824 (CELL SYSTEMS LTD ET AL.) 25 November 1987	
A	EP,A,0 312 114 (CULTOR LTD.) 19 April 1989	

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- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 OCTOBER 1992

Date of Mailing of this International Search Report

21. 10. 92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

REMPP G.L.E.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9201183  
SA 61473

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0246824	25-11-87	AU-B- 598260	21-06-90
		AU-A- 7299087	19-11-87
		JP-A- 63287479	24-11-88
-----			
EP-A-0312114	19-04-89	US-A- 4980277	25-12-90
		JP-A- 2000422	05-01-90
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